

## LAW OFFICES

**SUGHRUE, MION, ZINN, MACPEAK & SEAS, PLLC**

09/446634

2100 PENNSYLVANIA AVENUE, N.W.  
 WASHINGTON, D.C. 20037-3202  
 TELEPHONE (202) 293-7060  
 FACSIMILE (202) 293-7860

CALIFORNIA OFFICE

1010 EL CAMINO REAL  
 MENLO PARK, CA 94025  
 TELEPHONE (650) 325-5800  
 FACSIMILE (650) 325-6606

**ATTN: BOX PCT**

Assistant Commissioner for Patents  
 Washington, D.C. 20231

December 23, 1999

JAPAN OFFICE

TOEI NISHI SHIMBASHI BLDG. 4F  
 13-5 NISHI SHIMBASHI 1-CHOME  
 MINATO-KU, TOKYO 105, JAPAN  
 TELEPHONE (03) 3503-3760  
 FACSIMILE (03) 3503-3756

**ATTN: BOX PCT**

Re: Application of Hiroshi HAGIYA, Masashi MINAMI and Hisao TAJIMA  
**NOVEL PLASMID DNA COMPRISING REPORTER GENE DNA AND USE OF THE SAME**  
 Our Reference: Q57282  
 PCT/JP98/02785, filed June 23, 1998

Dear Sir:

Applicants herewith submit the attached papers for the purpose of entering the National stage under 35 U.S.C. § 371 and in accordance with Chapter II of the Patent Cooperation Treaty. Attached hereto is the application identified above which is a translation of PCT International Application No. PCT/JP98/02785, filed June 23, 1998, comprising the specification, claims, four (4) sheets of drawings, executed Declaration and Power of Attorney, Preliminary Amendment, International Search Report with PTO Form 1449, Notification Concerning Submission or Transmittal of Priority Document, Sequence Listing, foreign language International Preliminary Examination Report, executed Assignment and PTO Form 1595.

The Government filing fee is calculated as follows:

Total Claims	67 - 20 =	47 x \$18 =	\$ 846.00
Independent Claims	1 - 3 =	0 x \$78 =	\$ 000.00
Base Filing Fee	(\$840.00)		\$ 840.00
Multiple Dep. Claim Fee	(\$260.00)		\$ 260.00
<b>TOTAL FILING FEE</b>			<b>\$ 1946.00</b>
Recordation of Assignment Fee			\$ 40.00
<b>TOTAL U.S. GOVERNMENT FEE</b>			<b>\$ 1986.00</b>

Checks for the statutory filing fee of \$ 1946.00 and Assignment recordation fee of \$ 40.00 are attached. You are also directed and authorized to charge or credit any difference or overpayment to Deposit Account No. 19-4880. The Commissioner is hereby authorized to charge any fees under 37 C.F.R. 1.492; 1.16 and 1.17 which may be required during the entire pendency of the application to Deposit Account No. 19-4880. A duplicate copy of this transmittal letter is attached.

Priority is claimed from:

Japanese Patent ApplicationFiling Date

P. Hei. 9-171440

June 27, 1997

Respectfully submitted,  
 SUGHRUE, MION, ZINN, MACPEAK & SEAS  
 Attorneys for Applicant(s)

By Mark Boland  
 Mark Boland  
 Registration No. 32,197

MXB:tnj

09/446634

410 Rec'd PCT/PTO 23 DEC 1999

ATTN: BOX PCT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

Hiroshi HAGIYA, Masashi MINAMI and Hisao TAJIMA

Serial No.: NOT YET ASSIGNED

PCT/JP98/02785, filed June 23, 1998

Filed: December 23, 1999

For: NOVEL PLASMID DNA COMPRISING REPORTER GENE DNA AND USE OF THE SAME

PRELIMINARY AMENDMENT

ATTN: BOX PCT

Assistant Commissioner for Patents  
Washington, DC 20231

Sir:

Prior to examination of the above-identified application, please amend the above-mentioned application as follows:

IN THE SPECIFICATION:

Please renumber the Sequence Listing pages, presently numbered as pages 33-35, to read as pages 1-3, respectively, and then place them behind the Declaration and Power of Attorney.

IN THE CLAIMS:

Please renumber the Claims pages, presently numbered as pages 36-39, to read as pages 33-36.

IN THE ABSTRACT:

Please renumber the Abstract of the Disclosure, presently numbered as page 40, to read as page 37.

REMARKS

Applicants submit no questions of new matter should arise and entry is requested.

Respectfully submitted,



Mark Boland  
Registration No. 32,197

SUGHRUE, MION, ZINN, MACPEAK & SEAS  
2100 Pennsylvania Avenue, N.W.  
Washington, D.C. 20037-3202  
Tel: (202) 293-7060  
MXB:tnj

Date: December 23, 1999

410 Rec'd PCT/PTO 23 DEC 1999

## DESCRIPTION

NOVEL PLASMID DNA COMPRISING REPORTER GENE DNA  
AND USE OF THE SAME

## TECHNICAL FIELD

The invention relates to (i) a plasmid DNA comprising a novel reporter gene DNA, (ii) a transformant transformed with the above plasmid DNA and a DNA encoding a known effector protein, (iii) a therapeutic agent for a cancer or an autoimmune disease, comprising both of the above DNAs as active ingredients, and (iv) a method for detecting a ligand of an intracellular receptor, comprising using the above transformant.

More particularly, the invention relates to (i) a plasmid DNA comprising a novel reporter gene, namely a minimum essential promoter and a DNA encoding an amino acid sequence comprising a transmembrane region and a functional region of an Fas antigen for detecting a gene expression effect, in a region downstream of an enhancer element in which a responsive element of Gal4 protein which is a yeast basal transcription factor (hereinafter referred to as "UAS") is repeated several times, (ii) a transformant transformed with both of the above plasmid DNA and a DNA encoding a fusion protein in which an amino acid sequence comprising a ligand binding region of an intracellular receptor is linked to the C-terminal of an amino acid sequence comprising the DNA binding region of a known effector protein, namely Gal4 protein, (iii) a therapeutic agent for a cancer or an autoimmune disease, comprising both of the above DNAs as active

ingredients and (iv) a method for detecting an intracellular receptor ligand, comprising using the above transformant.

## BACKGROUND ART

Recently, receptors of humoral factors having important physiological activities, typified by cytokine and hormones, have been revealed by gene manipulation, and a large number of receptors have been isolated and identified. The examples include receptors of lipophilic hormones generally referred to as intracellular receptors. As a result of the analysis of amino acid sequences of various intracellular receptors, it was revealed that they are ligand-dependent transcription factors having a common basic structure and forming one of gene family. That is, they belong to a family including a steroid receptor, such as glucocorticoid receptor, progesterone receptor, mineral corticoid receptor, androgen receptor, estrogen receptor and the like; a retinoid receptors, such as retinoid X receptor, retinoic acid receptor and the like; a peroxisome proliferator-activated receptor; a vitamin D<sub>3</sub> receptor; and a thyroid hormone receptor. Since it has been revealed that functional regions, such as a ligand binding domain and a domain which recognizes a target DNA sequence and the like, are distinctively separated in these receptors (see *Science*, 240, 889 (1988)), they have been isolated based on the homology, though there are certain receptors whose ligands are still unknown.

Recently, a nuclear receptor, peroxisome proliferator-activated receptor (hereinafter referred to as a "PPAR receptor"), is drawing attention in the field of studies on transcription factors related to the expression and induction of fat cell differentiation marker genes. Regarding the PPAR receptor, cDNA has been

cloned from various animal species, and a plurality of isoform genes have been found including  $\alpha$ ,  $\delta$  and  $\gamma$  types in mammals. In addition, it is known that the  $\gamma$  type is expressed in fat tissues, immunocytes, adrenal gland, spleen and small intestine, and the  $\alpha$  type in liver and retinas, but the  $\delta$  type has no tissue specificity and is expressed ubiquitously.

On the other hand, Fas antigen is a membrane protein whose participation in programmed cell death, named apoptosis has been clearly shown. Yonehara *et al.* have prepared monoclonal antibodies for human cell surface antigens and obtained an anti-Fas antibody showing lethal activity upon various human cells (see *J. Exp. Med.*, 169, 1747 (1989)). cDNA of a cell surface molecule recognizable by the anti-Fas antibody has been isolated, and structure of the human Fas antigen has been determined (see *Cell*, 66, 233 (1991)). This Fas antigen is composed of 335 amino acid residues, and 16 amino acid residues in the N-terminus is assumed to be its signal peptide. A transmembrane region composed of 17 hydrophobic amino acid residues is present in a central position of the molecule, and it is considered that 157 amino acid residues in the N-terminus exist as its extracellular region and the C-terminal side sequence of 145 amino acid residues is its cytoplasmic region.

Since the structure of the Fas antigen is resemble in the structure of a receptor for tumor necrosis factor (TNF), it is assumed that the apoptosis of the Fas antigen occurs by a mechanism similar to the action of TNF. Functional regions of the Fas antigen have also been revealed gradually, and it has been found that the region essential for the signal transduction of apoptosis (functional region) is an amino acid sequence of the 175th to 304th positions (see *J. Biol. Chem.*, 268, 10932 (1993)). In addition, the amino acid sequence of mouse Fas

antigen has also been revealed (see *J. Immunology*, 148, 1274 (1992)), and it has a homology of 49.3% to the human Fas antigen as a whole. It is considered also that its functional region is an amino acid sequence of the 166th to 291st positions corresponding to the region of the human Fas antigen.

Receptors have a ligand binding region and a signalling transduction region. When a ligand is linked to the ligand binding region, the stereostructure of the signal transfer region is changed and a signal is transferred to other protein or DNA.

Recently, many studies have been conducted positively on the intercellular signal transduction by a fusion protein in which the ligand binding region of a receptor is linked to the signalling region of a different protein.

For example, it has been revealed that, in the case of cells transformed with a gene encoding a fusion protein of the ligand binding region of an estrogen receptor which is one of steroid receptors family, and the functional region of human cancer gene c-Myc, the cells are cancerated to abnormally proliferate by the stimulation of the ligand, estrogen (see *Nature*, 340, 66 (1989)).

Similar studies have been carried out on fusion proteins of the ligand binding region of each of the receptors for glucocorticoid, mineral corticoid and estrogen with the functional region of each of the proteins of E1A (adenovirus), c-Fos, v-Myb, C/EBP, v-Re1, GATA-1, 2 and 3, GAL4-VP16, Rev (HIV) and c-Ab1 (see *Cell*, 54, 1073 (1988), *Proc. Natl. Acad. Sci. USA*, 88, 5114 (1991), *EMBO J.*, 10, 3713 (1991), *Science*, 251, 288 (1991), *EMBO J.*, 11, 4641 (1992), *Genes Dev.* (in press), *Proc. Natl. Acad. Sci. USA*, 90, 1657 (1993), *ibid.*, 87, 7787 (1990) and *EMBO J.*, 12, 2809 (1993)), confirming that respective signals are transferred by the binding of ligands to their corresponding nuclear receptors.

Kakizuka *et al.* have reported a method in which the Fas antigen is used as a signal transduction protein for applying the above fusion protein to the treatment of a cancer (see JP-A-7-316200). In this method, an amino acid sequence of the 136th to 305th positions including not only the functional region but also the transmembrane region is fused with the ligand binding region of the intracellular receptor. As a result, a ligand capable of interacting with the amino acid sequence of the nuclear receptor ligand binding region can be detected effectively.

On the other hand, R.M. Evans *et al.* have reported a reporter assay method in which a reporter using an enzyme capable of converting a substrate into a chemiluminescent or visible dye product or introducing a substituent (e.g., luciferase,  $\beta$ -galactosidase, secretory alkaline phosphatase, or chloramphenicol acetyltransferase) in a region downstream of the Gal4 responsive element and a protein prepared by fusing the Gal4 DNA binding domain and the PPAR receptor ligand binding region are introduced into cells (see PCT International Application International Publication No. 9640128). Another assay method has also been reported in which, either by expressing an exogenous PPAR receptor protein or along with an endogenous PPAR receptor protein, the above reporter, with the proviso that in this case, the reporter is a reporter using the above enzyme in a region downstream of PPAR receptor responsive element (hereinafter referred to as "PPRE") are introduced (*Cell*, 83, 803 (1995)). These assay systems are detection systems which can evaluate the function of intracellular receptors as transcription factors.

However, since these methods require a certain period of time for the activity detection, it is difficult to carry out high speed screening of the effect of

ligands and compounds to be tested. In addition, since the introduction of DNA into cells is transient, it causes a problem in that the results cannot easily be compared due to insufficient stability among tests.

#### DISCLOSURE OF THE INVENTION

For carrying out high speed screening of compounds which bind to ligands of intracellular receptors as an object, the inventors of the invention have conducted intensive studies and found, as a result of the efforts, that the object can be attained using a reporter assay in which an Fas protein is expressed.

That is, an expression vector comprising a reporter using a functional region of an Fas structural protein and a DNA encoding a protein prepared by fusing a DNA binding region of Gal4 and a nuclear receptor (particularly PPAR receptor) ligand binding region was stably introduced into mouse fibroblast L929 cells. These cells were able to grow under such conditions that ligands or compounds did not bind to the amino acid sequence of the nuclear receptor ligand binding region, but cell death occurred due to expression induction of an Fas protein when the ligands or compounds bound to the sequence. Thereafter, we have succeeded in carrying out high speed screening of a compound which interacts with the ligand binding region of an intracellular receptor and can functionally increase or decrease the activity of transcription factor by separating the thus cell death-caused cells from intact cells, staining the intact cells with a dye, and measuring the absorbance of the stained cells.

The high throughput screening method with the high speed of the invention is a novel method which has been unknown completely.



In the method of the invention, the measurement is carried out by generating cell death through expression of an Fas protein using functional region as a reporter of an Fas protein, so that it is completely different from the method of R.M. Evans *et al.* using an enzyme which generates chemiluminescence (particularly luciferase) or forms a visible dye (particularly  $\beta$ -galactosidase) as the reporter and measuring the amount of expressed the enzyme (enzyme activity).

In comparing by the standard test methods, the method of R.M. Evans *et al.* requires an experimental period for 4 days at the shortest in which DNA is introduced into cells and serum stimulation is carried out on the first day, re-inoculation is optionally carried out, a compound to be tested is added after 46 to 48 hours (on the third day), and luciferase activity is measured 24 hours thereafter (on the final day). Also, since the introduction of DNA into cells is transient, it causes a problem in terms of stability and comparison between tests. An expression vector comprising a DNA encoding another enzyme may be co-introduced in order to compare each measurements, but the handling becomes complex. In addition, since the measured value greatly varies, the method using luciferase requires a large number of cases (generally requires an n number of 3 to 4). Alternatively, it is necessary to standardize the method with a simultaneously introduced internal standard gene (e.g.,  $\beta$ -galactosidase gene *etc.*) as described above.

On the other hand, the invention is a method of two-day schedule at the shortest, in which, when cells are prepared in advance, a compound to be tested is added on the first day, and the cells can be measured by a dye staining on the latter half of the next day (after 36 hours). Also, since L929 cells (the same clone) are used, comparison between tests can be made easily. In addition,

since the variation of values is small when measured by the method of the invention in which stably introduced cells are used, it does not require a large number of cases (generally, sufficient judgment can be made by an n number of 1 to 2).

Thus, according to the method of the invention, it is possible to carry out high speed evaluation of ligands and compounds which can change functions of intracellular receptors, and the handling *per se* is simple and easy. Also, since variation of measured values is small, data between tests can be easy to compare and are very stable. These advantages exert full abilities particularly in the high speed evaluation of multiple samples. In addition, since an absorbance measuring apparatus is attached to any generally and practically used automatic analyzers (robotic machine) as a standard measure, the method can be regarded as a robot-corresponding compound evaluation system. This is a fact which cannot be expected from the previous art but confirmed for the first time by experiments carried out by the present inventors. In addition, since the method of the invention require no enzyme substrate, sharp reduction of cost can be made.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph showing dose-dependent changes in the number of intact cells after treatment of each compound by carrying out a cell death assay using PPAR  $\gamma$  ligand-responsive L929 cells. In the drawing, (A) shows a case of PPAR  $\gamma$  ligand-responsive cells, and (B) shows a case of normal L929 cells.

Fig. 2 is a graph showing dose-dependent changes in the number of intact cells after treatment of each compound by carrying out a cell death assay

using PPAR  $\gamma$  ligand-responsive L929 cells. In the drawing, (A) shows a case of PPAR  $\gamma$  ligand-responsive cells, and (B) shows a case of normal L929 cells.

Fig. 3 is a graph showing dose-dependent changes in the number of intact cells after treatment of each compound by carrying out a cell death assay using PPAR  $\alpha$  ligand-responsive L929 cells. In the drawing, (A) shows a case of PPAR  $\alpha$  ligand-responsive cells, and (B) shows a case of normal L929 cells.

Fig. 4 is a graph showing dose-dependent changes in the number of intact cells after treatment of each compound by carrying out a cell death assay using PPAR  $\delta$  ligand-responsive L929 cells. In the drawing, (A) shows a case of PPAR  $\delta$  ligand-responsive cells, and (B) shows a case of normal L929 cells.

#### DETAILED DESCRIPTION OF THE INVENTION

The invention relates to

- (i) a novel plasmid DNA comprising a reporter gene DNA,
- (ii) a transformant transformed with the above plasmid DNA and a DNA encoding a known effector protein,
- (iii) a therapeutic agent for a cancer or autoimmune disease, comprising both of the above DNAs as active ingredients, and
- (iv) a method for detecting a ligand of an intracellular receptor, comprising using the transformant.

The Fas antigen and intracellular receptor for use in the invention are those which are originated from mammals, such as humans, monkeys, dogs, cats, mice, rats and guinea pigs. The Fas antigen and intracellular receptor are preferably originated from the same species, but those from the different species can also be used. It has been confirmed by the invention that the transfer of

signals can be effected when those which are originated from different species are used. For example, a combination of human Fas antigen with a human intracellular receptor, a combination of mouse Fas antigen with a human intracellular receptor, a combination of human Fas antigen with a rat intracellular receptor, a combination of mouse Fas antigen with a rat intracellular receptor, a combination of human Fas antigen with a mouse intracellular receptor or a combination of mouse Fas antigen with a mouse intracellular receptor can be used suitably.

As the amino acid sequence comprising the transmembrane region and the function expressing region of an Fas antigen and the amino acid sequence comprising the ligand binding region of an intracellular receptor for use in the invention, the portion corresponding to each region may be used alone, or the portion corresponding to each region may further contain a flanking region (a region which does not take part in the functional region; several tens, preferably 60 or less, of residues as an amino acid sequence) on its both termini (N-terminal and/or C-terminal). If desired, an amino acid sequence comprising a signal peptide region may be linked to the N-terminal of the transmembrane region of the Fas antigen. The amino acid sequence corresponding to each region may be not only a natural amino acid sequence but also its derivative in which at least one amino acid is deleted, substituted, added and/or inserted in a such extent that the original apoptosis function of the Fas antigen or the ligand binding function of the intracellular receptor is not spoiled.

Among the Fas antigens for use in the invention, the amino acid sequence comprising the transmembrane region and the function expressing region of the human Fas antigen is a sequence of the 145th Ser to the 319th Val,

and, in the case of the mouse Fas antigen, a sequence of the 136th Ser to the 305th Leu is preferably used. Also, as the amino acid sequence comprising a signal peptide region, a sequence of the -16th to 23rd positions of human Fas antigen or a sequence of the -21st to 14th positions of mouse Fas antigen is preferably used.

Examples of the intracellular receptor in the effector protein for use in the invention include a steroid receptor, such as a glucocorticoid receptor, a progesterone receptor, a mineral corticoid receptor, an androgen receptor, an estrogen receptor and the like; a retinoid receptor, such as retinoid X receptor, retinoic acid receptor and the like; a peroxisome proliferator-activated receptor; a vitamin D<sub>3</sub> receptor; and a thyroid hormone receptor. Among these, human or rat estrogen receptor; human or mouse retinoid X receptor  $\alpha$ , retinoid X receptor  $\beta$  or retinoid X receptor  $\gamma$ ; human or mouse retinoic acid receptor  $\alpha$ , retinoic acid receptor  $\beta$  or retinoic acid receptor  $\gamma$ ; human or mouse PPAR  $\alpha$  receptor; human or mouse PPAR  $\delta$  receptor; and human or mouse PPAR  $\gamma$  receptor are preferably used.

The ligand binding region of each receptor is already known (see *Science*, 240, 889 (1988); *Mol. Endocrinology*, 6, 1634 (1992); *Biochem. Biophys. Res. Commun.*, 224, 431 (1996); *J. Steroid Biochem. Molec. Biol.*, 51, 157 (1994); and *Proc. Natl. Acad. Sci. USA*, 91, 7355 (1994)), such as 528th to 777th positions in the human glucocorticoid receptor, the 680th to 934th positions in the human progesterone receptor, the 734th to 984th positions in the human mineral corticoid receptor, the 676th to 919th positions in the human androgen receptor, the 311th to 551st positions in the human estrogen receptor, the 307th to 557th positions in the rat estrogen receptor, the 225th to 462nd positions in the human retinoid X

receptor  $\alpha$ , the 297th to 526th positions in the human retinoid X receptor  $\beta$ , the 230th to 467th positions in the mouse retinoid X receptor  $\alpha$ , the 171st to 410th positions in the mouse retinoid X receptor  $\beta$ , the 229th to 463rd positions in the mouse retinoid X receptor  $\gamma$ , the 198th to 462nd positions in the human retinoic acid receptor  $\alpha$ , the 191st to 448th positions in the human retinoic acid receptor  $\beta$ , the 200th to 454th positions in the human retinoic acid receptor  $\gamma$ , the 198th to 462nd positions in the mouse retinoic acid receptor  $\alpha$ , the 190th to 448th positions in the mouse retinoic acid receptor  $\beta$ , the 200th to 458th positions in the mouse retinoic acid receptor  $\gamma$ , the 192nd to 427th positions in the human vitamin D<sub>3</sub> receptor, the 183rd to 410th positions in the human thyroid hormone receptor  $\alpha$ , the 232nd to 456th positions in the human thyroid hormone receptor  $\beta$ , the 280th to 478th positions in the human PPAR  $\gamma$  (human PPAR  $\gamma$ 1 subtype) receptor, the 278th to 475th positions in the mouse PPAR  $\gamma$  (mouse PPAR  $\gamma$ 1 subtype) receptor, the 273rd to 468th positions in human PPAR  $\alpha$  receptor, the 273rd to 468th positions in the mouse PPAR  $\alpha$  receptor, the 273rd to 468th positions in rat PPAR  $\alpha$  receptor, the 246th to 441st positions in the human PPAR  $\delta$  receptor, and the 245th to 440th positions in the mouse PPAR  $\delta$  receptor.

Preferred examples include a sequence of the 311th position to the 551st position of the human estrogen receptor, the 307th to 557th positions of the rat estrogen receptor, the 225th to 462nd positions of the human retinoid X receptor  $\alpha$ , the 297th to 526th positions of the human retinoid X receptor  $\beta$ , the 230th to 467th positions of the mouse retinoid X receptor  $\alpha$ , the 171st to 410th positions of the mouse retinoid X receptor  $\beta$ , the 229th to 463rd positions of the mouse retinoid X receptor  $\gamma$ , the 198th to 462nd positions of the human retinoic acid receptor  $\alpha$ , the 191st to 448th positions of the human retinoic acid receptor  $\beta$ ,

the 200th to 454th positions of the human retinoic acid receptor  $\gamma$ , the 198th to 462nd positions of the mouse retinoic acid receptor  $\alpha$ , the 190th to 448th positions of the mouse retinoic acid receptor  $\beta$ , the 200th to 458th positions of the mouse retinoic acid receptor  $\gamma$ , the 176th to 478th positions of the human PPAR  $\gamma$  (human PPAR  $\gamma$ 1 subtype) receptor, the 174th to 475th positions of the mouse PPAR  $\gamma$  (mouse PPAR  $\gamma$ 1 subtype) receptor, the 167th to 468th positions of the human PPAR  $\alpha$  receptor, the 167th to 468th positions of the mouse PPAR  $\alpha$  receptor, the 167th to 468th positions of the rat PPAR  $\alpha$  receptor, the 139th to 441st positions of the human PPAR  $\delta$  receptor, and the 138th to 440th positions of the mouse PPAR  $\delta$  receptor.

More preferred examples include a sequence of the 281st to 595th positions of the human estrogen receptor, the 286th to 600th positions of the rat estrogen receptor, the 176th to 462nd positions of the human retinoic acid receptor  $\alpha$ , the 177th to 458th positions of the mouse retinoic acid receptor  $\alpha$ , the 166th to the 478th positions of the human PPAR  $\gamma$  (human PPAR  $\gamma$ 1 subtype) receptor (corresponding to a sequence of the 194th to 506th positions of the human PPAR  $\gamma$ 2 subtype), the 164th to 475th positions of the mouse PPAR  $\gamma$  (mouse PPAR  $\gamma$ 1 subtype) receptor (corresponds to a sequence of the 194th to 505th positions of the mouse PPAR  $\gamma$ 2 subtype), the 157th to 468th positions of the human PPAR  $\alpha$  receptor, the 157th to 468th positions of the mouse PPAR  $\alpha$  receptor, the 157th to 468th positions of the rat PPAR  $\alpha$  receptor, the 129th to 441st positions of the human PPAR  $\delta$  receptor, and the 128th to 440th positions of the mouse PPAR  $\delta$  receptor.

Examples of more preferred effector protein for use in the invention include:

- (i) an effector protein in which an amino acid sequence of the 176th serine (Ser) to the 478th tyrosine (Tyr) of the human PPAR  $\gamma$ 1 receptor is linked to the C-terminal of an amino acid sequence of the 1st to 147th positions of the DNA binding region of Gal4 protein,
- (ii) an effector protein in which an amino acid sequence of the 174th serine (Ser) to the 475th tyrosine (Tyr) of the mouse PPAR  $\gamma$ 1 receptor is linked to the C-terminal of an amino acid sequence of the 1st to 147th positions of the DNA binding region of Gal4 protein,
- (iii) an effector protein in which an amino acid sequence of the 204th serine (Ser) to the 506th tyrosine (Tyr) of the human PPAR  $\gamma$ 2 receptor is linked to the C-terminal of an amino acid sequence of the 1st to 147th positions of the DNA binding region of Gal4 protein,
- (iv) an effector protein in which an amino acid sequence of the 204th serine (Ser) to the 505th tyrosine (Tyr) of the mouse PPAR  $\gamma$ 2 receptor is linked to the C-terminal of an amino acid sequence of the 1st to 147th positions of the DNA binding region of Gal4 protein,
- (v) an effector protein in which an amino acid sequence of the 167th serine (Ser) to the 468th tyrosine (Tyr) of the human PPAR  $\alpha$  receptor is linked to the C-terminal of an amino acid sequence of the 1st to 147th positions of the DNA binding region of Gal4 protein,
- (vi) an effector protein in which an amino acid sequence of the 167th serine (Ser) to the 468th tyrosine (Tyr) of the mouse PPAR  $\alpha$  receptor is linked to the C-terminal of an amino acid sequence of the 1st to 147th positions of the DNA binding region of Gal4 protein,



(vii) an effector protein in which an amino acid sequence of the 139th serine (Ser) to the 441st tyrosine (Tyr) of the human PPAR  $\delta$  receptor is linked to the C-terminal of an amino acid sequence of the 1st to 147th positions of the DNA binding region of Gal4 protein, and

(viii) an effector protein in which an amino acid sequence of the 138th serine (Ser) to the 440th tyrosine (Tyr) of the mouse PPAR  $\delta$  receptor is linked to the C-terminal of an amino acid sequence of the 1st to 147th positions of the DNA binding region of Gal4 protein.

Regarding the UAS which is a Gal4 protein responsive element contained as a repeating structure in the reporter plasmid of the invention, an already known sequence was used (see *Cell*, 83, 803 (1995) and *Cell*, 83, 813 (1995)). That is, a sequence in which a sequence of 5'-CGACGGAGTACTGT CCTCCG-3' is repeated several times, specifically at least 3 times, preferably 4 times, more preferably 5 times.

Examples of the promoter of the invention include SV40, HSV LTR, a metallothionein promoter, and a thymidine kinase (TK) promoter. A TK promoter is preferred. More specifically, it is preferred to use a sequence of the -727th to 56th positions of the mRNA of the TK promoter, when its transcription initiation point is regarded as the 1st position, as used in plasmid pTK $\beta$  (Clontech Laboratories, Cat. No. 6179-1) (corresponding to a sequence of the 165th to 945th positions in the pTK $\beta$  map). A sequence of the -105th to 51st positions of the mRNA of the TK promoter is more preferred.

DNAs encoding the reporter plasmid of the invention are also included in the invention. As well known, one amino acid is encoded by 1 to 6 codons (e.g., 1 codon for methionine (Met) and 6 codons for leucine (Leu)). Accordingly,

the nucleotide sequence of a DNA can be changed without changing its corresponding amino acid sequence.

Among DNAs encoding the reporter plasmid of the invention, a group of nucleotide sequences of all DNA sequences which result in the same Fas protein polypeptide are included in the invention. Productivity of the fusion protein may sometimes be improved by changing the nucleotide sequence.

The DNA encoding the reporter plasmid of the invention can be prepared in accordance with the following method.

That is, this method is carried out by

- (i) amplifying DNAs encoding amino acid sequences comprising necessary regions of the amino acid sequences of an Fas antigen and an intracellular receptor by the polymerase chain reaction (PCR), and
- (ii) inserting the PCR product obtained from the Fas antigen, and subsequently the PCR product obtained from the intracellular receptor, into an appropriate vector.

In detail, the step (i) is a step in which necessary parts for fusion are amplified by PCR. In the case of the Fas antigen, a nucleotide sequence encoding an amino acid sequence comprising the transmembrane region and the function expressing region is chemically synthesized and used as a primer. Also, in the case of the intracellular receptor, a nucleotide sequence encoding an amino acid sequence comprising the ligand binding region is chemically synthesized and used as a primer.

A specific restriction enzyme site is arranged on the 5'-end of each of the thus obtained primers. Regarding the template for PCR, mRNA isolated and purified from cells or a cell line of a corresponding mammal, such as human,

mouse or the like, can be used. The PCR is carried out by a known method. Since automatic PCR apparatuses have come into wide use recently, the apparatuses are suitably used.

In the step (ii), DNAs corresponding to the DNA binding region of Gal4 and the nuclear receptor ligand binding region are fused in an expression vector. As the plasmid vector for use in this step, a large number of vectors capable of functioning in *Escherichia coli* (e.g., pBR322) or *Bacillus subtilis* (e.g., pUB110) are known, and any of them can be used suitably. Also, it is possible to insert these fragments directly into an expression vector.

When expressed in mammal cells, the expression vector is prepared by inserting the PCR product of the Gal4 DNA binding region and then that of the intracellular receptor into a region downstream of an appropriate promoter (e.g., SV40 promoter, LTR promoter or metallothionein promoter) in an appropriate vector (e.g., a retrovirus vector, a papilloma virus vector, a vaccinia virus vector or an SV40 vector). Preferably, pCMX (described in *Cell*, 66, 663 (1991)) or pSV (described in *Anal. Biochem.*, 188, 245 (1990)) is used.

Subsequently, in the step (iii), a reporter plasmid is produced. The plasmid vector for use in this step may be any one of those which function in *E. coli* or *B. subtilis* as described above. It can also be inserted directly into the expression vector. A plasmid comprising a DNA in which a repeating structure of Gal4 responsive element, a herpes simplex virus thymidine kinase promoter and a DNA encoding an Fas structural protein are located in a tandem from the 5' region upstream can have the purpose depending on whether its origin is a vector which can function in *E. coli* or mammal cells. As described above, it may further contain an *ori* region, a promoter controlling factor and at least one selective

marker gene. When mammal cells are used, test cells which die in an Fas protein expression-dependent manner can be obtained by transforming appropriate mammal cells using the above plasmid capable of functioning in mammal cells and culturing the resulting transformant in an appropriate medium.

When the expression is carried out in mammal cells, a PCR product of a nucleotide sequence encoding the signal peptide region of an Fas antigen can be optionally inserted into a site immediately downstream of the promoter.

Examples of the replication or expression vector comprising the DNA of the invention include a plasmid, a virus and a phage vector comprising an *ori* region, and optionally a promoter for the expression of the above DNA and a controlling factor of the promoter or the like. The vector may further contain at least one selective marker gene (e.g., neomycin resistant gene, blasticidin S resistant gene).

Furthermore, as the protein expression system of the invention, the objective fusion protein can be produced by transforming appropriate mammal cells (e.g., monkey COS-7 cells, Chinese hamster CHO cells, mouse L cells, mouse fibroblasts, human cancer cells and the like) with the above expression vector which functions in mammal cells, and culturing the resulting transformant in an appropriate medium. The thus obtained polypeptide can be isolated and purified by generally used biochemical means.

The host cells transformed with a replication or expression vector comprising the DNA of the invention are included in the invention.

## INDUSTRIAL APPLICABILITY

The thus obtained plasmid DNA of the invention can be used as a therapeutic agent for a cancers and an autoimmune disease. That is, an expression vector comprising the plasmid DNA of the invention is topically administered to the focal part of a cancer or autoimmune disease by a targeting method (e.g., by its inclusion in liposomes). This vector penetrates into cancer cells of the focal part and reaches genes. Thereafter, when a ligand corresponding to the intracellular receptor which constitutes a part of the effector protein of the invention (for example, 9-cis-retinoic acid when a retinoid X receptor is used as the receptor, or vitamin A when a retinoic acid receptor is used) is administered, the effector is activated and the Fas protein is expressed. The signal of apoptosis is transduced by the Fas protein produced in the cancer cells, thereby the cells become extinct.

Furthermore, the plasmid DNA of the invention can be used broadly as a screening method of an agonist or an antagonist for an intracellular receptor. That is, a sample to be tested is added to cells into which an expression vector comprising the DNA encoding the effector protein (an expression vector DNA comprising a DNA encoding the ligand binding region of various nuclear receptors in a region downstream of the Gal4 DNA binding region) and the reporter DNA for use in the present invention has been inserted. Death of the cells indicates that the sample to be tested has agonist activity, and survival of the cells in the presence of an agonist reveals that the sample has antagonist activity.

According to the screening method of the invention, high speed evaluation of ligands and compounds capable of changing functions of intracellular receptors can be conducted. Since the effects (results) are observed as cell

death, the judgment is easy and the handling *per se* is also simple and easy. In addition, when a DNA encoding the ligand binding region of various nuclear receptors is arranged in a region downstream of the Gal4 DNA binding region as an effector protein, it becomes a method generally used which can screen compounds that act upon nuclear receptors including orphan receptors whose ligands are unknown. As a matter of course, it is possible to search for physiological ligands of orphan nuclear receptors. Specifically, it can be applied to the identification of ligands or their purification from fractions having ligand activities by adding a physiological ligand candidate to be expected or a partially fractionated sample of serum to the above cells and observing the presence or absence of cell death.

Also, since variation of values measured by the method of the invention is small, data between tests can be easy to compare and are very stable. These advantages exert full abilities particularly in the high speed evaluation of multiple samples. In addition, since an absorbance measuring apparatus is attached to any generally and practically used automatic analyzers (robotic machine) as a standard measure, the method can be regarded as a robot-corresponding compound evaluation system. Moreover, since the screening method of the invention does not require enzyme substrate, sharp reduction of cost can be made.

#### BEST MODE FOR CARRYING OUT THE INVENTION

The invention is described further in detail and specifically by the following examples, though the invention is not restricted thereto as a matter of course.

#### Example 1: Preparation of PCR product of mouse Fas antigen

The following primers were prepared based on the structure of the mouse Fas antigen in accordance with the method reported by Fukunaga *et al.* (see *J. Immunology*, 148, 1274 (1992)) or Kakizuka *et al.* (see JP-A-7-316200).

F1: A sense primer, 5'-CCAAGCTTGGCGACCAGCAATACAAACTGCAGGAAAC-3' (SEQ ID NO:1), corresponding to the structure of an amino acid sequence of the 131st to 143rd positions, namely Pro Cys Thr Ala Thr Ser Asn Thr Asn Cys Arg Lys Gln, in which a restriction enzyme *Hind*III site was introduced into a part of the 5' side, was synthesized.

R1: An antisense primer, 5'-TCAGGATCCAGACATTGTCCTTCATTTCATT-3' (SEQ ID NO:2), corresponding to an amino acid sequence of the 298th to 306th positions (C-terminal), namely Asn Glu Asn Gln Gly Gln Cys Leu Glu, and a portion of the 3' non-translation region, in which a *Bam*HI site was introduced into a part of the 3' side, was synthesized.

Using mRNAs obtained from a mouse T cell line, RLM-11 (*Cell*, 68, 1109 (1992)), as a template, and F1 and R1 as PCR primers, RT-PCR was carried out using a thermal sequencer (GeneAmp PCR System 9600, Perkin Elmer) under conditions of 180 seconds x 1 at 95°C → (60 seconds at 95°C, 60 seconds at 55°C, 60 seconds at 72°C) x 15 → 180 seconds at 72°C. As a result, a fragment of about 520 bp (hereinafter referred to as "MFas") was obtained and inserted into the multi-cloning site, *Hind*III-*Bam*HI restriction enzyme site, of pBluescript (trade name, Promega), hereinafter referred to as "pBSMFas".

Example 2: Preparation of PCR product of human PPAR  $\alpha$ ,  $\gamma$  or  $\delta$  receptor

The following primers were prepared based on the structure of human PPAR  $\alpha$ ,  $\gamma$  or  $\delta$  receptor described by R. Mukherjee *et al.* (see *J. Steroid Biochem. Molec. Biol.*, 51, 157 (1994)); M.E. Greene *et al.* (see *Gene Expression*, 4, 281 (1995)); A. Elbrecht *et al.* (see *Biochem. Biophys. Res. Commun.*, 224, 431 (1996)); or Schmidt (see *Mol. Endocrinol.*, 6, 1134 (1992).

F2: A sense primer, 5'-AACCAGCACCATCTGGTCGCGATGGT-3' (SEQ ID NO:3), corresponding to the 5' non-translation region and N-terminal amino acids Met<sup>1</sup> – Val<sup>2</sup> of the human PPAR  $\alpha$ , was synthesized.

R2: An antisense primer, 5'-AGGTGTGGCTGATCTGAAGGAACTCA-3' (SEQ ID NO:4), corresponding to the 3' non-translation region of the human PPAR  $\alpha$  and a amino acid termination codon, was synthesized.

F3: A sense primer, 5'-AGAAATGACCATGGTTGACACAGAGA-3' (SEQ ID NO:5), corresponding to the 5' non-translation region and N-terminal amino acids Met<sup>1</sup> – Met<sup>8</sup> of the human PPAR  $\gamma$  ( $\gamma$ 1 subtype), was synthesized.

R3: An antisense primer, 5'-AAATGTTGGCAGTGGCTCAGGACTCT-3' (SEQ ID NO:6), corresponding to the 3' non-translation region of the human PPAR  $\gamma$  ( $\gamma$ 1 subtype), was synthesized.

F4: A sense primer, 5'-AGATCAGCCATGGAGCAGCCACAGGA-3' (SEQ ID NO:7), corresponding to the 5' non-translation region and N-terminal amino acids Met<sup>1</sup> – Glu<sup>6</sup> of the human PPAR  $\delta$ , was synthesized.

R4: An antisense primer, 5'-ATTGGAGTCTGCAGGGAGGCCTGGGT-3' (SEQ ID NO:8), corresponding to the 3' non-translation region of the human PPAR  $\delta$ , was synthesized.



Using Superscript Human Liver cDNA Library (trade name, Cat. No. 10422-012, manufactured by GIBCO BRL) as cDNA library, and F2 and R2, F3 and R3 or F4 and R4 as PCR primers, PCR was carried out using a thermal sequencer (GeneAmp PCR System 9600, manufactured by Perkin Elmer) under conditions of 120 seconds x 1 at 95°C → (60 seconds at 95°C, 90 seconds at 60°C, 120 seconds at 72°C) x 30 → 180 seconds at 72°C. As a result, the human PPAR  $\alpha$  of 1450 bp, the human PPAR  $\gamma$  of 1469 bp and the human PPAR  $\delta$  of 1367 bp were obtained, and they were inserted into a T-vector (pT7Blue-T vector, Cat. No. 69836-1) manufactured by Novagen to confirm their complete nucleotide sequences.

Example 3: Effector protein expression system; Construction of expression vector comprising cDNA encoding fusion protein (Gal4 chimeric receptor protein) of Gal4 protein and nuclear receptor ligand binding region

A vector capable of expressing Gal4 chimeric receptor protein as a structural gene under control of SV40 promoter was constructed using Pica Gene Basic Vector 2 (trade name, PGBV2, Cat. No. 309-04821, manufactured by Toyo Ink) as a basic vector. That is, this was completed by excising a luciferase structural gene of PGBV2 using restriction enzymes *NcoI* and *XbaI* and inserting Gal4 chimeric receptor protein-encoding cDNA prepared in the following manner.

(1) Insertion of DNA encoding DNA binding region of Gal4 transcription factor into expression vector

As cDNA of the DNA binding region of a yeast basal transcription factor Gal4 protein, a DNA encoding an amino acid sequence of the 1st to 147th positions of Gal4 was amplified by PCR under the same conditions of Example 2

using pGBT9 (manufactured by Clontech, Cat. No. K1605-A) as the template. The following F5 and R5 were used as primers.

F5: A sense primer, 5'-GCAAGCTTCACCATGAAGCTACTGTCTTC TATCGAAC-3' (SEQ ID NO:9), corresponding to an N-terminal side amino acid sequence of the 1st to 8th positions of the DNA binding region of Gal4, in which a *HindIII* site and, in order to obtain effective protein expression in mammal cells, a kozak sequence were added to a part of the 5' side, was synthesized.

R5: An antisense primer, 5'-AGCCATGGCCGGCGATACAGTCAACT GTCTTTG-3' (SEQ ID NO:10), corresponding to a C-terminal side amino acid sequence of the 141st to 147th positions of the DNA binding region of Gal4, in which an *NcoI* site was added to a part of the 5' side, was synthesized, and the thus synthesized primer was amplified by PCR under the same conditions of Example 2. As a result, an amplified DNA of about 465 bp, after confirmation of its complete nucleotide sequence, was recombined with the *HindIII/NcoI* site in the PGBV2 vector (hereinafter referred to as "pGVgal").

(2) Preparation of DNA encoding human PPAR  $\gamma$  ligand binding region and construction of Gal4-human PPAR  $\gamma$  chimeric receptor protein expression vector

Using the complete human PPAR  $\gamma$ -encoding cDNA isolated from a human liver cDNA library in accordance with the procedure of Example 2 as a template and synthesizing the following primers, a DNA encoding a sequence of Ser<sup>176</sup> to Tyr<sup>478</sup> including the human PPAR  $\gamma$  ligand binding region was amplified by PCR under the same conditions of Example 1.

F6: A sense primer, 5'-GCCATGGCTCCTAAGAAGAAGCGTAAGG TAGGATCCCATAATGCCATCAGGTTTGGGCGGAT-3' (SEQ ID NO:11), corresponding to a sequence of Ser<sup>176</sup> to Met<sup>185</sup>, in which an *NcoI* site, an SV40 T

antigen-originated nuclear transport signal (Ala Pro Lys Lys Lys Arg Lys Val Gly) and a *Bam*HI site were arranged in this order on the 5' side, was synthesized.

R6: An antisense primer, 5'-CCTCTAGACTAGCTGGCATAGTCGGG CACGTCGTAGGGGTAGTCGACGTACAAGTCCTTGTAGATCTCC-3' (SEQ ID NO:12), corresponding to a sequence of Glu<sup>472</sup> to Tyr<sup>478</sup>, in which an *Sal*I site, an influenza hemagglutinin epitope (Tyr Pro Tyr Asp Val Pro Asp Tyr Ala) as an epitope tag sequence for use in the detection of expressed protein, a translation termination codon and an *Xba*I site were arranged in this order, was synthesized.

The luciferase structural gene in the above pGVgal was excised out both from the *Xba*I site of the region downstream of luciferase structural gene and the *Nco*I site at the region downstream of the DNA encoding the Gal4 DNA binding region, and replaced by a DNA encoding the human PPAR  $\gamma$  ligand binding region, thereby obtaining the effector protein expression vector of interest (hereinafter referred to as "pGVgal/ $\gamma$ LBD").

(3) Preparation of DNA encoding human PPAR  $\alpha$  ligand binding region and construction of Gal4-human PPAR  $\alpha$  chimeric receptor protein expression vector

A PPAR  $\alpha$  ligand binding region structural gene was synthesized by synthesizing the following primers comprising the human PPAR  $\alpha$  ligand binding region and carrying out PCR amplification under the same conditions of Example 2. The thus obtained PPAR  $\alpha$  ligand binding region structural gene was inserted into the *Bam*HI-*Sal*I restriction enzyme site of the pGVgal/ $\gamma$ LBD vector synthesized above which had been digested with these restriction enzymes to selectively excise the PPAR  $\gamma$  ligand binding region structural gene to construct a Gal4-human PPAR  $\alpha$  chimeric receptor protein expression vector (hereinafter referred to as "pGVgal/ $\alpha$ LBD").

F7: A sense primer, 5'-CACGGATCCCACAACGCGATTTCGTTTTGGACGA-3' (SEQ ID NO:13), corresponding to an amino acid sequence of Ser<sup>167</sup> to Arg<sup>175</sup> of the human PPAR  $\alpha$  and having a *Bam*HI site in its 5' side, was synthesized.

R7: An antisense primer, 5'-ATGGTCGACGTACATGTCCTGTAGATCTCTCTG-3' (SEQ ID NO:14), corresponding to an amino acid sequence of Gln<sup>461</sup> to Tyr<sup>468</sup> of the human PPAR  $\alpha$  and having an *Sal*I site in its 5' side, was synthesized.

(4) Preparation of DNA encoding human PPAR  $\delta$  ligand binding region and construction of Gal4-human PPAR  $\delta$  chimeric receptor protein expression vector

A PPAR  $\delta$  ligand binding region structural gene was synthesized by synthesizing the following primers comprising the human PPAR  $\delta$  ligand binding region and carrying out PCR amplification under the same conditions of Example 2. The thus obtained PPAR  $\delta$  ligand binding region structural gene was inserted into the *Bam*HI-*Sal*I restriction enzyme site of the pGVgal/ $\gamma$ LBD vector synthesized above which had been digested with these restriction enzymes to selectively excise the PPAR  $\gamma$  ligand binding region structural gene to construct the Gal4-human PPAR  $\delta$  chimeric receptor protein expression vector (hereinafter referred to as "pGVgal/ $\delta$ LBD").

F8: A sense primer, 5'-CACGGATCCCACAACGCTATCCGTTTTGGTCGG-3' (SEQ ID NO:15), corresponding to an amino acid sequence of Ser<sup>139</sup> to Arg<sup>147</sup> of the human PPAR  $\delta$  and having a *Bam*HI site in its 5' side, was synthesized.

R8: An antisense primer, 5'-ATGGTCGACGTACATGTCCTTGTAGATCTCTCTG-3' (SEQ ID NO:16), corresponding to an amino acid sequence of

Gln<sup>434</sup> to Tyr<sup>441</sup> of the human PPAR  $\delta$  and having an *Sal*I site in its 5' side, was synthesized.

Example 4: Reporter gene; Construction of reporter plasmid in which TK promoter and MFas structural gene are arranged in region downstream of Gal4 transcription factor responsive element

An expression vector pTK $\beta$  (manufactured by Clontech, Cat. No. 6179-1) under control of herpes simplex virus thymidine kinase (TK) promoter was used. *Not*I sites upstream and downstream of the  $\beta$ -galactosidase structural gene of pTK $\beta$  were digested, and both termini are smooth-ended. Next, a DNA encoding the MFas protein, as a DNA to be inserted, was excised from pBSMFas by using restriction enzymes *Hind*III and *Bam*HI and blunt-ended. Both of the DNAs were ligated to select a clone in which the MFas structural gene was connected to the region downstream of TK promoter in the forward direction. The thus obtained plasmid pTK-MFas of about 4400 bp contains a DNA encoding MFas protein under control of the TK promoter. Next, the *Sal*I site located in the region upstream of the TK promoter of plasmid pTK-MFas was digested and blunt-ended, and then a multi-cloning site was inserted as a linker DNA (SEQ ID NO:17, *Eco*RI-*Sal*I-*Kpn*I-*Eco*RV-*Sac*I-*Not*I):

5'-GAATTCGTCGACGGTACCGATATCGAGCTCGCGGCCGC-3'

3'-CTTAAGCAGCTGCCATGGCTATAGCTCGAGCGCCGGCG-5'

to obtain pTK-MFas-ML1(5'-*Eco*RI-*Sal*I-*Kpn*I-*Eco*RV-*Sac*I-*Not*I-3').

An enhancer region was produced by inserting a Gal4 responsive element, 5'-T (C G A C G G A G T A C T G T C C T C C G)  $\times$  4 AGCT-3' (SEQ ID NO:18), having 4 repetition of a basal unit (UAS) and *Sal*I site/*Sac*I site on both

ends, into the *Sall*–*Sacl* site inside the pTK–MFas–ML1 multi-cloning site. As a result, p4xUAS–TK–MFas (about 4600 bp) in which the 4 x UAS, TK promoter and MFas structural gene were arranged in this order was constructed.

Each of the thus obtained expression vector pSVgal/αLBD, pSVgal/γLBD and pSVgal/δLBD, the reporter plasmid and pSV2bsr (manufactured by Kaken Pharmaceutical, Cat. No. KK–500) were subjected to linearization by the treatment with a restriction enzyme *AatII* or *NotI* or not treated (circular form), respectively, and introduced at a quantitative ratio of 1 : 1 : 0.1 into mouse fibroblast L929 using Lipofect AMINE (manufactured by GIBCO, Cat. No. 18324–012). Stably transformed cells were selected in accordance with the method (blasticidin S selection) described in *Exp. Cell Res.*, 197, 229 (1991).

#### Example 5: Selection of ligand responsive clone

After 1 to 2 weeks of selection, observed colonies were subjected to cell cloning by limiting dilution and grown. For example, each subclone was suspended in Eagle's MEM containing 10% fetal calf serum (FCS) in a density of  $2 \times 10^4$  cells/100  $\mu$ l, dispensed into wells of 96 well microtiter plate and cultured at 37°C for 4 to 6 hours in an atmosphere of 5% carbon dioxide. Cells which die significantly in the presence of a ligand were selected using CS–045 and BRL–49653 known as PPAR  $\gamma$  ligands (see *Cell*, 83, 863 (1995), *Endocrinology*, 137, 4189 (1996) and *J. Med. Chem.*, 39, 665 (1996)), carbacyclin and ETYA known as PPAR  $\alpha$  ligands (cf. *J. Steroid Biochem. Molec. Biol.*, 51, 157 (1994), *Cell Structure and Function*, 18, 267 (1993), *Gene & Development*, 10, 974 (1996) and *Eur. J. Biochem.*, 233, 242 (1996)), carbacyclin known as PPAR  $\delta$  ligand (see *Gene & Development*, 10, 974 (1996)) and a compound disclosed by Merck (see

the compound of Example 7 in PCT International Application International Publication No. 9728149). That is, each sample of these compounds having two times higher concentration than their final concentration 10  $\mu$ M was added to each of the above culture wells, and the cells were again cultured for 40 hours. After discarding the supernatant, the resulting plate was soaked in a Crystal Violet solution (0.75% Crystal Violet–0.25% NaCl–1.75% formaldehyde in 50% ethanol) at room temperature for 20 minutes and then washed twice with phosphate buffered saline (PBS), thereby effecting staining of survived cells (see *Cell*, 66, 233 (1991)). Absorbance of each well of the plate was directly measured at 570 nm using a microplate reader.

Example 6: Cell death assay using L929 cells stably introduced with plasmid encoding PPAR  $\gamma$ ,  $\alpha$  or  $\delta$  ligand

Each of the clones obtained in Example 5 having the highest responsive ability was cultured by the above method, cell death of the thus cultured cells was induced by the above compounds by the above method and then the survived cells were stained to measure the absorbance. The cell death appeared significantly after 24 hours and was completed during 36 to 40 hours by reaching to the plateau stage.

Dose-dependent changes in the number of intact cells after 40 hours of treatment of the PPAR  $\gamma$  ligand-responsive clone with CS-045 and BRL-49653 are shown in Fig. 1 (A), and dose-dependent changes in the number of intact cells after treatment of control cells (L929) with these compounds in the same manner are shown in Fig. 1 (B). Dose-dependent changes in the number of intact cells after 40 hours of treatment of the PPAR  $\gamma$  ligand-responsive clone with carbacyclin

and Wy-14643 are shown in Fig. 2 (A), and dose-dependent changes in the number of intact cells after treatment of control cells (L929) with these compounds in the same manner are shown in Fig. 2 (B).

Also, dose-dependent changes in the number of intact cells after 40 hours of treatment of the PPAR  $\alpha$  ligand-responsive clone with carbacyclin, ETYA, Wy-14634 and CS-045 are shown in Fig. 3 (A), and dose-dependent changes in the number of intact cells after treatment of control cells (L929) with these compounds in the same manner are shown in Fig. 3 (B).

Also, dose-dependent changes in the number of intact cells after 40 hours of treatment of the PPAR  $\alpha$  ligand-responsive clone with carbacyclin, the compound of Merck, ETYA and CS-045 are shown in Fig. 4 (A), and dose-dependent changes in the number of intact cells after treatment of control cells (L929) with these compounds in the same manner are shown in Fig. 4 (B).

Also, the ratio of the number of intact cells when each compound was replaced by a solvent was expressed as 100% in each drawing.

As apparent from Fig. 1, in the case of the cells into which the expression vector of effector protein Gal4-human PPAR  $\gamma$  chimeric receptor protein was stably introduced, CS-045 and BRL-49653 known as PPAR  $\gamma$  agonists did not show direct toxicity upon normal L920 cells, but caused significant death of responsive cells in a dose-dependent manner. In addition, although they are the same PPAR  $\gamma$  agonist, the compound having higher binding affinity for PPAR  $\gamma$  protein or larger transcription activation ability (cf. *Biochem. Biophys. Res. Commun.*, 224, 431 (1996)) showed remarkable cell killing action at a lower concentration.



As apparent from Fig. 2, carbacyclin and Wy-14643 known as a PPAR  $\alpha$  agonists do not cause cell death at respective concentrations of 10  $\mu$ M or less and 100  $\mu$ M or less which do not exert toxicity upon the cells. Particularly, at a concentration of 10  $\mu$ M or less by which carbacyclin does not show PPAR  $\gamma$  agonist activity (cf. *Genes & Development*, 10, 974 (1996)), it does not cause cell death in the PPAR  $\gamma$  ligand responsive cells.

As apparent from Fig. 3, carbacyclin, ETYA and Wy-14643 known as PPAR  $\alpha$  agonists cause dose-dependent cell death. On the other hand, CS-045 known as a PPAR  $\gamma$  agonist does not cause cell death at a concentration of 10  $\mu$ M or less which does not show toxicity. In addition, although the former three are the same PPAR  $\alpha$  agonist, a compound having higher binding affinity for PPAR  $\alpha$  protein or larger transcription activation ability showed remarkable cell killing action at a lower concentration.

As apparent from Fig. 4, carbacyclin and Merck's compound known as PPAR  $\delta$  agonists cause dose-dependent cell death. On the other hand, CS-045 known as a PPAR  $\gamma$  agonist and ETYA known as a PPAR  $\alpha$  agonist do not cause cell death at a concentration of 10  $\mu$ M or less which does not show toxicity. In addition, though the former two compounds are the same PPAR  $\delta$  agonist, the compound having higher binding affinity for PPAR  $\delta$  protein or larger transcription activation ability showed remarkable cell killing action at a lower concentration.

Based on the results shown in Figs. 1, 2, 3 and 4, it was revealed that, when cell death is mediated by the over-expression of a functional MFas peptide, cell death of PPAR  $\gamma$  ligand-responsive cells occurs in good correlation with the action strength of the PPAR  $\gamma$  ligand, that of PPAR  $\alpha$  ligand-responsive cells

correlatively with the action strength of the PPAR  $\alpha$  ligand, and that of PPAR  $\delta$  ligand-responsive cells with the PPAR  $\delta$  ligand. In addition, the cell death occurred when not only the PPAR but also other nuclear receptors were used and when the PPAR of not only human but also other animal origin was used. When the reporter assay of the invention is used, nuclear receptor ligands can be evaluated easily and specifically by obtaining highly ligand-responsive clones prepared by stably introducing respective plasmid therein. Additionally, since the amount of compounds to be tested effective for their evaluation by the reporter assay markedly coincides with the range of concentrations showing the cell-killing action in the system of the invention, this is an excellent method by which potency of respective ligands can be evaluated simultaneously.

## SEQUENCE LISTING

SEQ ID NO:1  
SEQUENCE LENGTH: 35  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
SEQUENCE:  
CCAAGCTTGG CGACCAGCAA TACAACTGCAG GAAAC

SEQ ID NO:2  
SEQUENCE LENGTH: 32  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
SEQUENCE:  
TCAGGATCCA GACATTGTCC TTCATTTTCA TT

SEQ ID NO:3  
SEQUENCE LENGTH: 26  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
SEQUENCE:  
AACCAGCACC ATCTGGTCGC GATGGT

SEQ ID NO:4  
SEQUENCE LENGTH: 26  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
SEQUENCE:  
AGGTGTGGCT GATCTGAAGG AACTCA

SEQ ID NO:5  
SEQUENCE LENGTH: 26  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
SEQUENCE:  
AGAAATGACC ATGGTTGACA CAGAGA

SEQ ID NO:6  
SEQUENCE LENGTH: 26  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
SEQUENCE:  
AAATGTTGGC AGTGGCTCAG GACTCT

SEQ ID NO:7  
SEQUENCE LENGTH: 26

SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
SEQUENCE:  
AGATCAGCCA TGGAGCAGCC ACAGGA

SEQ ID NO:8  
SEQUENCE LENGTH: 26  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
SEQUENCE:  
ATTGGAGTCT GCAGGGAGGC CTGGGT

SEQ ID NO:9  
SEQUENCE LENGTH: 37  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
SEQUENCE:  
GCAAGCTTCA CCATGAAGCT ACTGTCTTCT ATCGAAC

SEQ ID NO:10  
SEQUENCE LENGTH: 33  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
SEQUENCE:  
AGCCATGGCC GGCGATACAG TCAACTGTCT TTG

SEQ ID NO:11  
SEQUENCE LENGTH: 62  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
SEQUENCE:  
GCCATGGCTC CTAAGAAGAA GCGTAAGGTA GGATCCCATA ATGCCATCAG GTTTGGGCGG 60  
AT 62

SEQ ID NO:12  
SEQUENCE LENGTH: 69  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
SEQUENCE:  
CCTCTAGACT AGCTGGCATA GTCGGGCACG TCGTAGGGGT AGTCGACGTA CAAGTCCTTG 60  
TAGATCTCC 69

SEQ ID NO:13  
SEQUENCE LENGTH: 33  
SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single  
TOPOLOGY: linear  
SEQUENCE  
CACGGATCCC ACAACGCGAT TCGTTTTGGA CGA

SEQ ID NO:14  
SEQUENCE LENGTH: 31  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
SEQUENCE:  
ATGGTCGACG TACATGTCCCTG TAGATCTCCT G

SEQ ID NO:15  
SEQUENCE LENGTH: 33  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
SEQUENCE:  
CACGGATCCC ACAACGCTAT CCGTTTTGGT CGG

SEQ ID NO:16  
SEQUENCE LENGTH: 33  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
SEQUENCE:  
ATGGTCGACG TACATGTCCT TG TAGATCTC CTG

SEQ ID NO:17  
SEQUENCE LENGTH: 38  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: double  
TOPOLOGY: linear  
SEQUENCE:  
GAATTCGTCG ACGGTACCGA TATCGAGCTC GCGGCCGC  
CTTAAGCAGC TGCCATGGCT ATAGCTCGAG CGCCGGCG

SEQ ID NO 18  
SEQUENCE LENGTH: 85  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
SEQUENCE:  
TCGACGGAGT ACTGTCCTCC GCGACGGAGT ACTGTCCTCC GCGACGGAGT ACTGTCCTCC 60  
GCGACGGAGT ACTGTCCTCC GAGCT 85

## CLAIMS

1. A plasmid DNA comprising a promoter and a DNA encoding an amino acid sequence comprising a transmembrane region and a functional region of an Fas antigen, in a region downstream of a responsive element of Gal4 protein.

2. The plasmid DNA according to claim 1, wherein the amino acid sequence comprising the transmembrane region and the functional region of the Fas antigen is an amino acid sequence of the 136th to 305th positions of mouse Fas antigen or an amino acid sequence of the 145th to 319th positions of human Fas antigen.

3. The plasmid DNA according to claim 1 or 2, wherein an amino acid sequence comprising a signal peptide region of the Fas antigen is linked to the N-terminal of the amino acid sequence comprising the transmembrane region and the functional region of the Fas antigen.

4. The plasmid DNA according to claim 3, wherein the amino acid sequence comprising the signal peptide region of the Fas antigen is an amino acid sequence of the -21st to 14th positions of mouse Fas antigen or an amino acid sequence of the -16th to 23rd positions of human Fas antigen.

5. A transformant transformed with both of the plasmid DNA of claim 1 and a DNA encoding an effector protein.

6. The transformant according to claim 5, wherein the effector protein is a fusion protein in which an amino acid sequence comprising a ligand binding region of a nuclear receptor is linked to the C-terminal of an amino acid sequence comprising a DNA binding region of Gal4 protein.

7. The transformant according to claim 6, wherein the nuclear receptor is a steroid receptor.

8. The transformant according to claim 6, wherein the nuclear receptor is a PPAR  $\alpha$  receptor.

9. The transformant according to claim 8, wherein the amino acid sequence comprising the ligand binding region of the nuclear receptor is an amino acid sequence of the 167th to 468th positions of human PPAR  $\alpha$  receptor or an amino acid sequence of the 167th to 468th positions of mouse PPAR  $\alpha$  receptor.

10. The transformant according to claim 6, wherein the nuclear receptor is a PPAR  $\delta$  receptor.

11. The transformant according to claim 10, wherein the amino acid sequence comprising the ligand binding region of the nuclear receptor is an amino acid sequence of the 139th to 441st positions of human PPAR  $\delta$  receptor or an amino acid sequence of the 138th to 440th positions of mouse PPAR  $\delta$  receptor.

12. The transformant according to claim 6, wherein the nuclear receptor is a PPAR  $\gamma$  receptor.

13. The transformant according to claim 12, wherein the amino acid sequence comprising the ligand binding region of the nuclear receptor is an amino acid sequence of the 176th to 478th positions of human PPAR  $\gamma$ 1 receptor, an amino acid sequence of the 174th to 475th positions of mouse PPAR  $\gamma$ 1 receptor, an amino acid sequence of the 204th to 506th positions of human PPAR  $\gamma$ 2 receptor, or an amino acid sequence of the 204th to 505th positions of mouse PPAR  $\gamma$ 2 receptor.

14. The transformant according to claim 6, wherein the nuclear receptor is a retinoid X receptor.

15. The transformant according to claim 14, wherein the amino acid sequence comprising the ligand binding region of the nuclear receptor is an amino acid sequence of the 225th to 462nd positions of human retinoid X receptor  $\alpha$ , an amino acid sequence of the 297th to 526th positions of human retinoid X receptor  $\beta$ , an amino acid sequence of the 230 to 467th positions of mouse retinoid X receptor  $\alpha$ , an amino acid sequence of the 171st to 410 positions of mouse retinoid X receptor  $\beta$ , or an amino acid sequence of the 229th to 463rd positions of mouse retinoid X receptor  $\gamma$ .

16. The transformant according to claim 6, wherein the nuclear receptor is a retinoic acid receptor.

17. The transformant according to claim 16, wherein the amino acid sequence comprising the ligand binding region of the nuclear receptor is an amino acid sequence of the 198th to 462nd positions of human retinoic acid receptor  $\alpha$ , an amino acid sequence of the 191st to 448th positions of human retinoic acid receptor  $\beta$ , an amino acid sequence of the 200th to 454th positions of human retinoic acid receptor  $\gamma$ , an amino acid sequence of the 198th to 462nd positions of mouse retinoic acid receptor  $\alpha$ , an amino acid sequence of the 190th to 448th positions of the mouse retinoic acid receptor  $\beta$ , or an amino acid sequence of the 200th to 458th positions of mouse retinoic acid receptor  $\gamma$ .

18. The transformant according to claim 6, wherein the nuclear receptor is a vitamin D<sub>3</sub> receptor.

19. The transformant according to claim 6, wherein the nuclear receptor is a thyroid hormone receptor.



20. A therapeutic agent for a cancer or an autoimmune disease, comprising the plasmid DNA of any one of claims 1 to 4 and a DNA encoding an effector protein.

21. A therapeutic agent for a cancer or an autoimmune disease, comprising a DNA for transforming the transformant of any one of claims 5 to 19 as an active ingredient.

22. A method for screening an agonist or an antagonist for a intracellular receptor, comprising using the transformant of any one of claims 5 to 19.

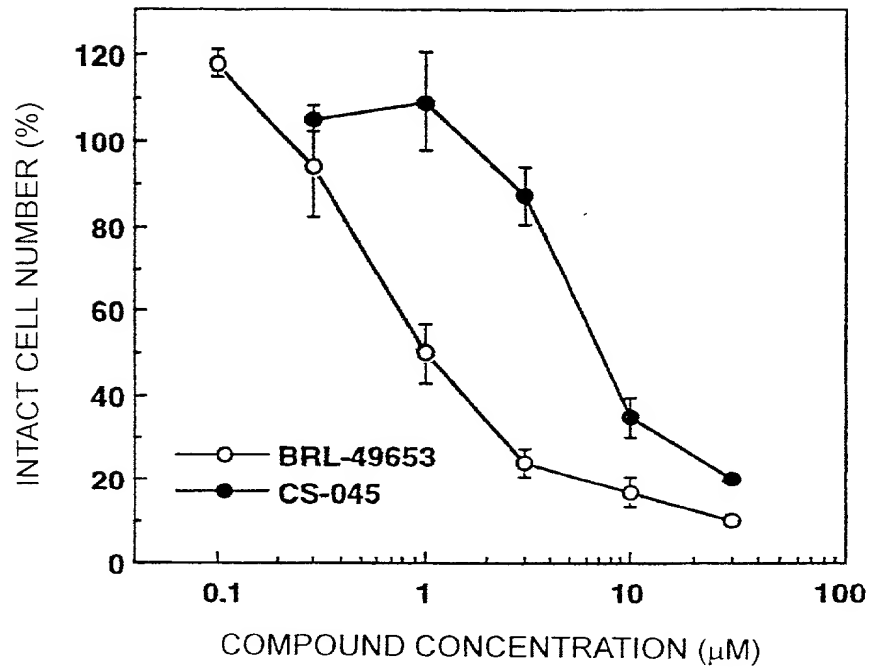
23. A method for screening an agonist or an antagonist for a PPAR  $\alpha$  receptor, a PPAR  $\gamma$  receptor or a PPAR  $\delta$  receptor, wherein the transformant of any one of claims 5 to 19 is mouse fibroblast L929 or human cancer cell HeLa.

## ABSTRACT

A plasmid DNA comprising a novel reporter gene DNA; a transformant transformed with the above plasmid DNA and a DNA encoding a known effector protein; a therapeutic agent for a cancer or an autoimmune disease, comprising both of the above DNAs as active ingredients; and a method for detecting a ligand of an intracellular receptor, comprising using the above transformant.

FIG. 1

(A)



(B)

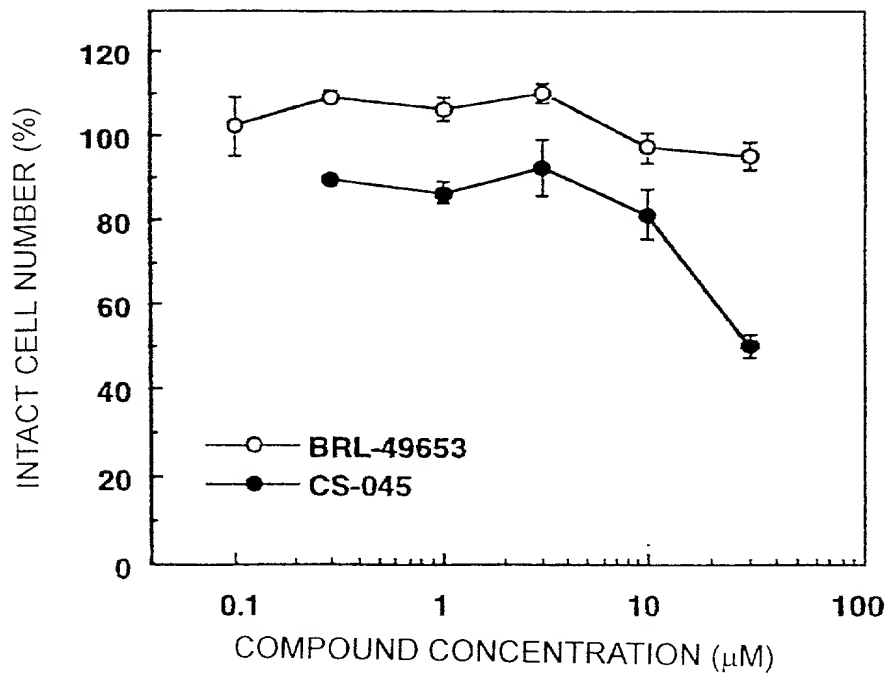
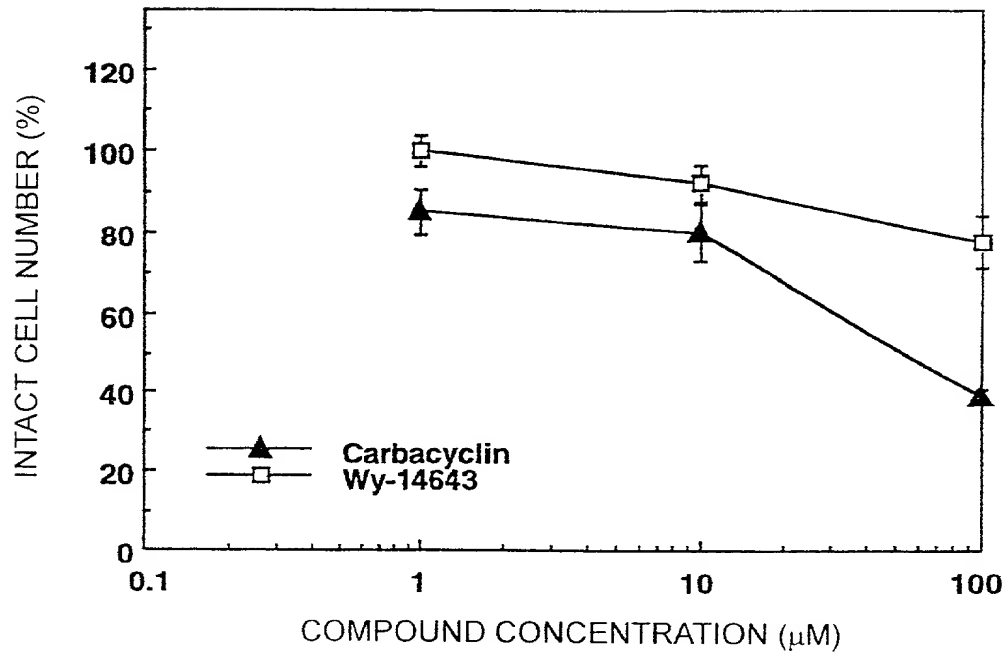


FIG. 2

(A)



(B)

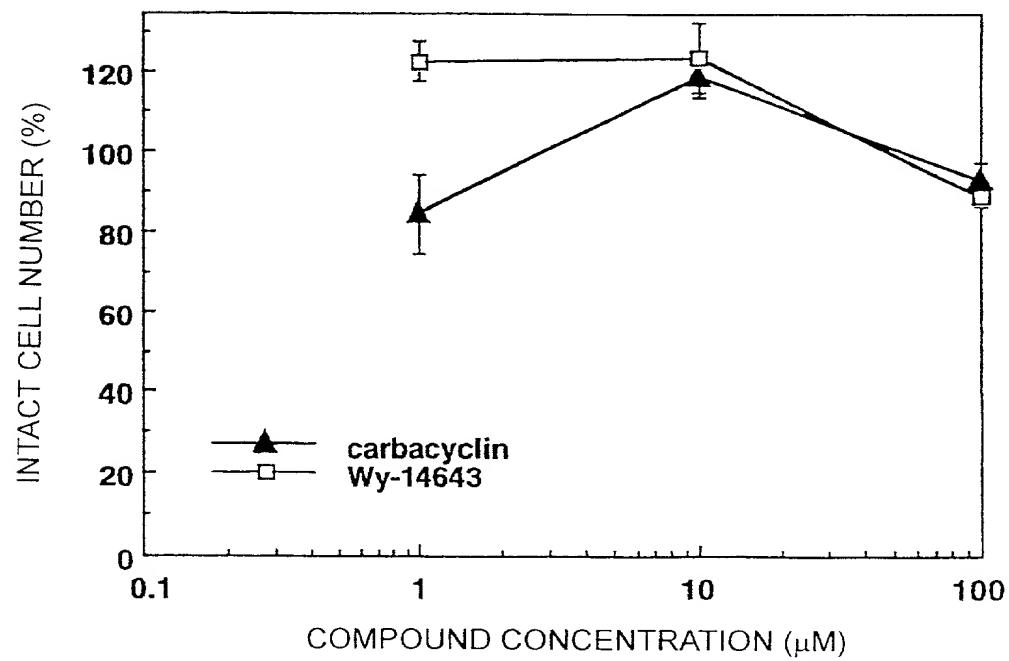
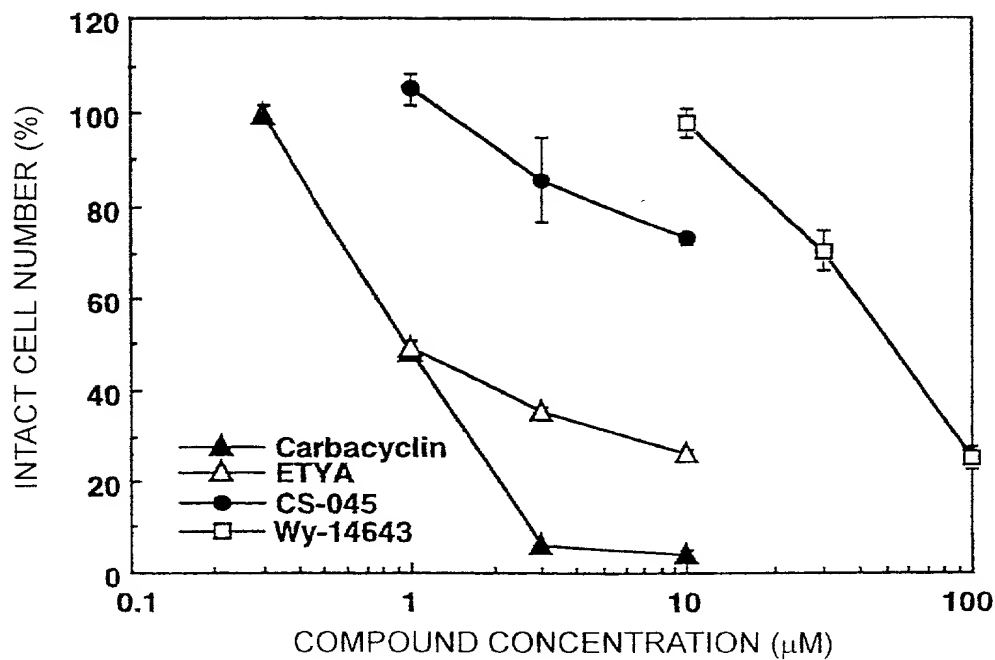


FIG. 3

(A)



(B)

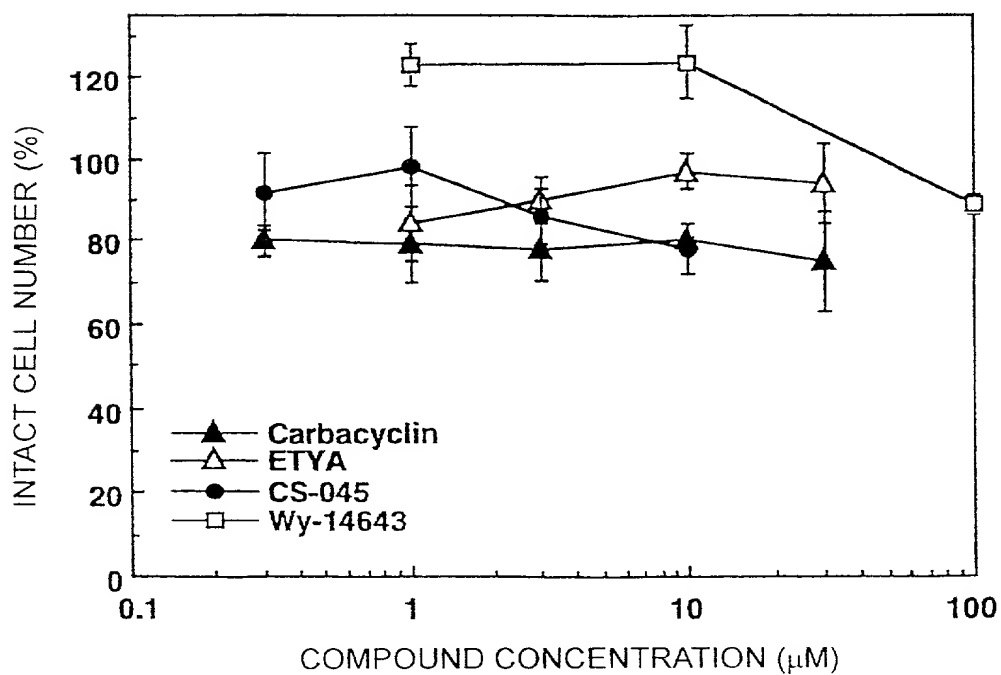
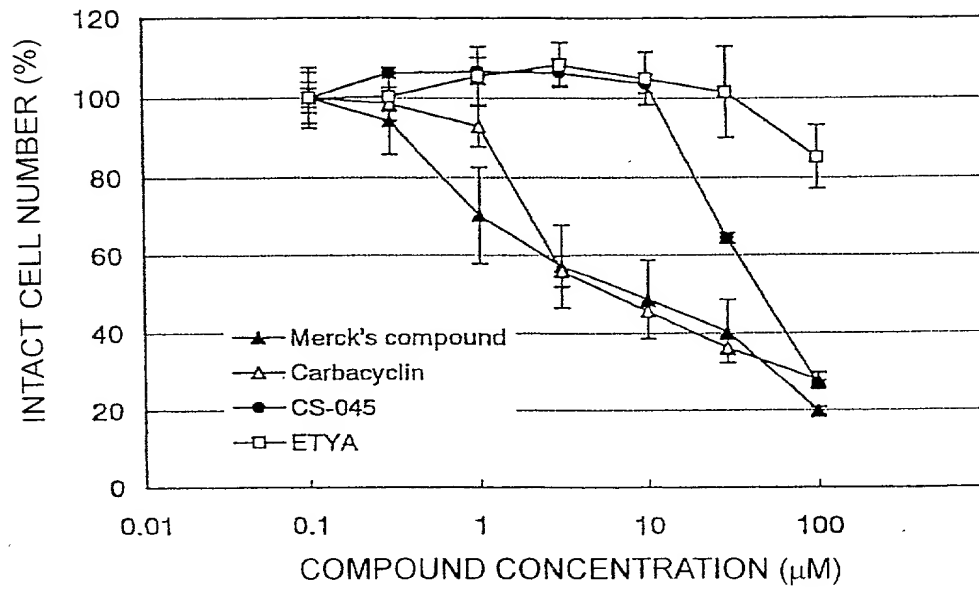
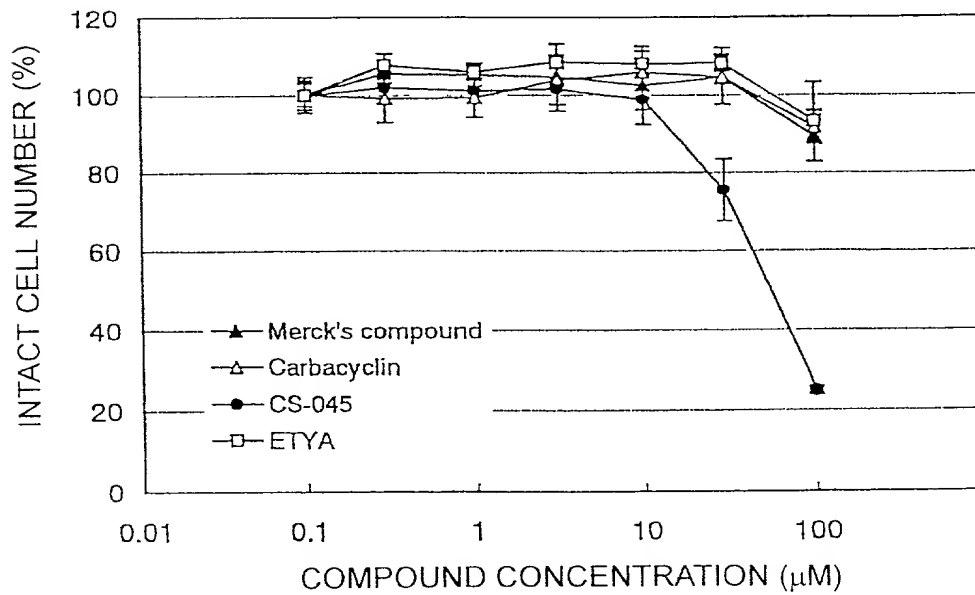


FIG. 4

(A)



(B)



# DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

## NOVEL PLASMID DNA COMPRISING REPORTER GENE DNA AND USE OF THE SAME

the specification of which is attached hereto unless the following box is checked:

☒ was filed on June 23, 1998 as United States Application Number or PCT International Application Number PCT/JP98/02785 and was amended on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information of which is material to the patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Not Claimed

<u>P. Hei. 9-171440</u> (Number)	<u>Japan</u> (Country)	<u>27/June/1997</u> (Day/Month/Year Filed)	<input type="checkbox"/>
_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	<input type="checkbox"/>
_____ (Number)	_____ (Country)	_____ (Day/Month/Year Filed)	<input type="checkbox"/>

I hereby claim the benefits under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

(Application Number)

(Filing Date)

I hereby claim the benefits under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

(Application Number)

(Filing Date)

(Status - patented, pending, abandoned)

(Application Number)

(Filing Date)

(Status - patented, pending, abandoned)

I hereby appoint John H. Mion, Reg. No. 18,879; Thomas J. Macpeak, Reg. No. 19,292; Robert J. Seas, Jr., Reg. No. 21,092; Darryl Mexic, Reg. No. 23,063; Robert V. Sloan, Reg. No. 22,775; Peter D. Olexy, Reg. No. 24,513; J. Frank Osha, Reg. No. 24,625; Waddell A. Biggart, Reg. No. 24,861; Louis Gubinsky, Reg. No. 24,835; Neil B. Siegel, Reg. No. 25,200; David J. Cushing, Reg. No. 28,703; John R. Inge, Reg. No. 26,916; Joseph J. Ruch, Jr., Reg. No. 26,577; Sheldon I. Landsman, Reg. No. 25,430; Richard C. Turner, Reg. No. 29,710; Howard L. Bernstein, Reg. No. 25,665; Alan J. Kasper, Reg. No. 25,426; Kenneth J. Burchfiel, Reg. No. 31,333; Gordon Kit, Reg. No. 30,764; Susan J. Mack, Reg. No. 30,951; Frank L. Bernstein, Reg. No. 31,484; Mark Boland, Reg. No. 32,197; William H. Mandir, Reg. No. 32,156; Scott M. Daniels, Reg. No. 32,562; Brian W. Hannon, Reg. No. 32,778; Abraham J. Rosner, Reg. No. 33,276; Bruce E. Kramer, Reg. No. 33,725; Paul F. Neils, Reg. No. 33,102; Brett S. Sylvester, Reg. No. 32,765 and Robert M. Masters, Reg. No. 35,603; my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and request that all correspondence about the application be addressed to SUGHRUE, MION, ZINN, MACPEAK & SEAS, PLLC, 2100 Pennsylvania Avenue, N.W., Washington, D.C. 20037.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date December 10, 1999

First Inventor Hiroshi

HAGIYA

First Name

Last Name

Residence Osaka,

Signature Hiroshi

Hagiya

Japan

Post Office Address c/o Minase Research Institute, ONO Pharmaceutical Co., Ltd., 1-1, Sakurai 3-chome, Shimamoto-cho, Mishima-gun, OSAKA 618-8585 JAPAN SPX

Citizenship Japan

Date December 10, 1999

Second Inventor Masashi

MINAMI

First Name

Last Name

Residence Osaka,

Signature Masashi

Minami

Japan

Post Office Address c/o Minase Research Institute, ONO Pharmaceutical Co., Ltd., 1-1, Sakurai 3-chome, Shimamoto-cho, Mishima-gun, OSAKA 618-8585 JAPAN SPX

Citizenship Japan

300

Date December 10, 1999

Third Inventor Hisao

TAJIMA

First Name

Last Name

Residence Osaka,

Signature

Hisao

Tajima

Japan

Post Office Address c/o Minase Research Institute, ONO Pharmaceutical  
Co., Ltd., 1-1, Sakurai 3-chome, Shimamoto-cho,  
Mishima-gun, OSAKA 618-8585 JAPAN JPX

Citizenship Japan

Date \_\_\_\_\_

Fourth Inventor \_\_\_\_\_

First Name

Last Name

Residence \_\_\_\_\_

Signature \_\_\_\_\_

Post Office Address \_\_\_\_\_

Citizenship \_\_\_\_\_

Date \_\_\_\_\_

Fifth Inventor \_\_\_\_\_

First Name

Last Name

Residence \_\_\_\_\_

Signature \_\_\_\_\_

Post Office Address \_\_\_\_\_

Citizenship \_\_\_\_\_

Date \_\_\_\_\_

Sixth Inventor \_\_\_\_\_

First Name

Last Name

Residence \_\_\_\_\_

Signature \_\_\_\_\_

Post Office Address \_\_\_\_\_

Citizenship \_\_\_\_\_

Date \_\_\_\_\_

Seventh Inventor \_\_\_\_\_

First Name

Last Name

Residence \_\_\_\_\_

Signature \_\_\_\_\_

Post Office Address \_\_\_\_\_

Citizenship \_\_\_\_\_

Date \_\_\_\_\_

Eighth Inventor \_\_\_\_\_

First Name

Last Name

Residence \_\_\_\_\_

Signature \_\_\_\_\_

Post Office Address \_\_\_\_\_

Citizenship \_\_\_\_\_

(Supply similar information for ninth and subsequent joint inventors.)